CNS CHLORIDE MODULATION AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit, under 35 U.S.C. § 119(e), of United States provisional patent application Serial No. 60/470,885 filed May 16, 2003, which is hereby incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to the modulation of anion levels in a central nervous system (CNS) neural cell, and particularly relates to the modulation of CNS intracellular chloride levels and uses thereof for treating, preventing, diagnosing and prognosticating pain.

BACKGROUND OF THE INVENTION

The need for new and improved methods and agents for pain treatment is a significant ongoing concern in medicine. Acute pain, e.g. related to injury or disease, can be severe and have critical effects on patient recovery. An even greater concern is chronic pain, which affects a large proportion of the population, causing not only significant discomfort, but can result in low self-esteem, depression, anger, and can interfere with or completely prevent a sufferer from typical daily activities.

While a number of studies have been done in this area, many mechanisms and pathways involved in pain sensation remain poorly understood. As in the case of the sensation of various stimuli, it has been suggested that pain sensation is related to altered neuronal excitability.

Ion cotransport has in some cases been thought to play a role in the processing of certain stimuli. For example, Howard et al. (28) have demonstrated that mice

generated with a targeted deletion of the Slc12a6 gene, which encodes the KCC3 exporter, exhibit features of agenisis of the corpus callosum, including a locomotor deficit, peripheral neuropathy and a sensorimotor gating deficit. Sung et al. (29) report that in mice where there is a disruption of the Slc12a2 gene, which encodes the NKCC1 cotransporter, sensitivity to thermal stimulus is greatly reduced, compared to both wild-type and heterozygous ($NKCC1^{+/-}$) mice.

There remains a need to better define the mechanisms involved in pain sensation to provide new strategies of therapeutic intervention in this regard.

SUMMARY OF THE INVENTION

This invention relates to pain and methods of treating, preventing, diagnosing and prognosticating such pain. This invention also relates to pain associated with neuropathic pain and CNS dysfunction. This invention also relates to methods of decreasing an intracellular chloride level in a central nervous system (CNS) neural cell.

According to one aspect, the invention provides a method of treating or preventing pain in a subject, the method comprising decreasing an intracellular chloride level in a central nervous system (CNS) neural cell of the subject. In an embodiment, the method comprises modulating the activity or expression of a chloride transporter in the CNS cell, thereby to decrease the chloride level. In a further embodiment, the chloride transporter is KCC2 the method comprises increasing KCC2 activity or expression. In another embodiment, the CNS neural cell is a spinal cord neural cell. In yet another embodiment, the signal of the pain originates in a peripheral nervous system (PNS) cell or sensory fiber

transsynaptic to the CNS neural cell. In still another embodiment, the pain is neuropathic pain, and in further embodiments the neuropathic pain is associated with a nerve or tract injury or is selected from the group consisting of somatic and visceral pain. In yet another embodiment, the pain is selected from the group consisting of chronic inflammatory pain, pain associated with arthritis, fibromyalgia, back pain, cancer-associated pain, pain associated with digestive disease, pain associated with Crohn's disease, pain associated with autoimmune disease, pain associated with endocrine disease, pain associated with diabetic neuropathy, phantom limb pain, spontaneous pain, chronic post-surgical pain, chronic temporomandibular pain, causalgia, post-herpetic neuralgia, AIDS-related pain, complex regional pain syndromes type I and II, trigeminal neuralgia, chronic back pain, pain associated with spinal cord injury and recurrent acute pain.

In an embodiment, the method comprises administering to the subject a compound capable of decreasing the intracellular chloride level in the CNS cell. In yet another embodiment, the compound is capable of modulating the activity or expression of a chloride transporter in the CNS cell. In yet a further embodiment, the chloride transporter is KCC2, and yet further, the compound is capable of increasing KCC2 activity or expression. In another embodiment, the compound is an inhibitor of TrkB, such as K-252a or an anti-TrkB antibody. In another embodiment, the compound is an inhibitor of cyclic AMP-dependent kinase (PKA) (e.g. H-89). In another embodiment, the compound is an inhibitor of calmodulin-dependant kinase (CAM kinase), and further, it is KN-93. In an embodiment, KCC2 comprises an amino acid sequence substantially identical to a sequence

selected from the group consisting of SEQ ID NO: 2, 4, 6 and a fragment thereof.

According to another aspect of the present invention, there is provided a composition for the treatment or the prevention of pain in a subject, the composition comprising a compound capable of decreasing an intracellular chloride level in a CNS neural cell; and a pharmaceutically acceptable carrier. In an embodiment, the compound is capable of modulating the activity or expression of a chloride transporter in the CNS neural cell. In a further embodiment, the chloride transporter is KCC2, and further, the compound is capable of increasing KCC2 activity or expression.

According to still another aspect of the invention, there is provided a commercial package comprising the composition described herein together with instructions for its use in the treatment or prevention of pain.

According to yet another aspect of the invention, there is provided a commercial package comprising a compound capable of decreasing an intracellular chloride level in a CNS neural cell together with instructions for its use the treatment or prevention of pain. In an embodiment, the compound is capable of modulating the activity or expression of a chloride transporter in said CNS neural cell. In a further embodiment, the chloride transporter is KCC2, and further, the compound is capable of increasing said KCC2 activity or expression.

According to a further aspect of the present invention, there is provided use of the composition described herein for the treatment or prevention of pain and/or for the preparation of a medicament for the treatment or prevention of pain.

According to yet a further aspect of the present invention, there is provided use of a compound capable of decreasing an intracellular chloride level in a CNS neural cell for the treatment or prevention of pain and/or for the preparation of a medicament for the treatment or prevention of pain. In an embodiment, the compound is capable of modulating the activity or expression of a chloride transporter in said CNS cell. In a further embodiment, the chloride transporter is KCC2, and further, the compound is capable of increasing KCC2 activity or expression. In another embodiment, the compound is an inhibitor of TrkB, and further, it is selected from the group consisting of K-252a and an anti-TrkB antibody. In another embodiment, the compound is an inhibitor of cyclic AMP-dependent kinase (PKA), and further, it is H-89. In another embodiment, the compound is an inhibitor of calmodulin-dependant kinase, and further, it is KN-93.

According to still a further aspect of the invention, there is provided a method of identifying or characterizing a compound for treatment or prevention of pain, the method comprising contacting a test compound with a CNS-derived cell; and determining whether the intracellular chloride level is decreased in the presence of the test compound; wherein the decrease is an indication that the test compound may be used for treatment or prevention of pain.

According to another aspect of the present invention, there is provided a method of identifying or characterizing a compound for treatment or prevention of pain, the method comprising contacting a test compound with a CNS-derived cell expressing a chloride transporter; and determining whether activity or expression of the chloride transporter is modulated in the presence of the test compound in such a way that the level intracellular chloride is

decreased; wherein the modulation is an indication that the test compound may be used for treatment or prevention of pain. In an embodiment, the chloride transporter is KCC2, and further, the method comprises determining whether said KCC2 expression or activity is increased in the presence of the test compound and the modulation is an increase. In another embodiment, KCC2 activity is determined by measuring a parameter selected from the group consisting of potassium transport, chloride transport, intracellular chloride level and anion reversal potential. In still another embodiment, the pain is selected from the group consisting of chronic inflammatory pain, pain associated with arthritis, fibromyalgia, back pain, cancer-associated pain, pain associated with digestive disease, pain associated with Crohn's disease, pain associated with autoimmune disease, pain associated with endocrine disease, pain associated with diabetic neuropathy, phantom limb pain, spontaneous pain, chronic post-surgical pain, chronic temporomandibular pain, causalgia, post-herpetic neuralgia, AIDS-related pain, complex regional pain syndromes type I and II, trigeminal neuralgia, chronic back pain, pain associated with spinal cord injury and recurrent acute pain.

According to yet another aspect of the present invention, there is provided a method of identifying or characterizing a compound for treatment or prevention of pain, said method comprising contacting a test compound with a CNS-derived cell comprising a first nucleic acid comprising a transcriptionally regulatory element normally associated with a chloride transporter gene, operably linked to a second nucleic acid comprising a reporter gene capable of encoding a reporter protein; and determining whether reporter gene expression or reporter protein activity is modulated in the presence of the test compound; wherein the modulation in

reporter gene expression or reporter protein activity being an indication that the test compound may be used for treatment or prevention of pain. In a further embodiment, the chloride transporter is KCC2, and further, the reporter gene expression or reporter protein activity is increased in the presence of the test compound.

According to one aspect of the present invention, there is provided a method for decreasing nociception in a subject, the method comprising decreasing intracellular chloride in a CNS neural cell of the subject. In an embodiment, the method comprises modulating chloride transporter activity or expression in the CNS neural cell. In a further embodiment, the chloride transporter is KCC2, and further, the method comprises increasing KCC2 activity or expression. In another embodiment, the method further comprises contacting the CNS neural cell with a compound capable of increasing KCC2 activity or expression. In yet another embodiment, the compound is an inhibitor of TrkB, and further, it is selected from the group consisting of K-252a and an anti-TrkB antibody. In still another embodiment, the compound is an inhibitor of cyclic AMP-dependent kinase (PKA), and further, it is H-89. In yet another embodiment, the compound is an inhibitor of calmodulin-dependant kinase, and further, it is KN-93. In still another embodiment, KCC2 comprises an amino acid sequence substantially identical to a sequence selected from the group consisting of SEQ ID NO: 2, 4, 6 and a fragment thereof.

According to another aspect of the invention, there is provided a method for diagnosing or prognosticating pain associated with CNS dysfunction in a subject experiencing pain, the method comprising determining whether a test CNS intracellular chloride level is increased relative to a corresponding control chloride level; wherein the increase is

an indication that the subject is experiencing pain associated with CNS dysfunction. In an embodiment, the method further comprises determining whether CNS chloride transporter activity or expression is modulated relative to a control transporter activity or expression. In another embodiment, the chloride transporter is KCC2, and further, the method comprises determining whether KCC2 activity or expression is decreased relative to the control activity or expression. In still another embodiment, the control intracellular chloride level is selected from the group consisting of an established standard; a corresponding intracellular chloride level determined in the subject at an earlier time; a corresponding intracellular chloride level determined in the subject when the subject is experiencing less pain or substantially no pain; and a corresponding intracellular chloride level determined in a control subject experiencing less pain or substantially no pain. In yet another embodiment, the control activity or expression is selected from the group consisting of an established standard of KCC2 activity or expression; a corresponding level of KCC2 activity or expression determined in the subject at an earlier time; a corresponding level of KCC2 activity or expression determined in the subject when the subject is experiencing less pain or substantially no pain; and a corresponding level of KCC2 activity or expression determined in a control subject experiencing less pain or substantially no pain. In a further embodiment, KCC2 activity is determined by measuring a parameter selected from the group consisting of potassium transport, chloride transport, intracellular chloride level and anion reversal potential. In still a further embodiment, the intracellular chloride level is determined by administering an indicator compound indicative of chloride level to the subject such that it is

contacted with a CNS neural cell of the subject; and assessing an *in vivo* signal associated with the indicator compound. In yet another embodiment, the pain associated with CNS dysfunction is neuropathic pain. In still yet another embodiment, the indicator compound is a radionuclide, and further, it is selected from the group consisting of ^{201}Tl, ^{99}Tcm-tetrofosmin, ^{99}Tcm-MIBI, ^{99}Tcm-HMPAO and ^{36}Cl. In still another embodiment, the *in vivo* signal is assessed by an imaging technique. In yet still another embodiment, the *in vivo* signal is the retention index of the indicator compound. In a further embodiment, the imaging technique is selected from the group consisting of single photon emission computed tomography, positron emission tomography and magnetic resonance imaging. In yet a further embodiment, the indicator compound is indicative of KCC2 expression, and further, it is an antibody directed against KCC2.

According to yet another aspect of the invention, there is provided a method of treating pain associated with CNS dysfunction in a subject, the method comprising diagnosing or prognosticating, according to the methods described herein, pain associated with CNS dysfunction in the subject; and decreasing an intracellular chloride level in a CNS cell of the subject.

In an embodiment, the above-mentioned subject is a mammal, in a further embodiment, a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Peripheral nerve injury (PNI) induced a collapse of the ∇_{anion} in Lamina I (LI) neurons in the ipsilateral superficial dorsal horn (SDH). **a)** Chronic constriction injury of the sciatic nerve (n = 23), but not sham surgery (n = 11), caused a significant reduction in the 50% nociceptive

withdrawal threshold to mechanical stimulation of the hindpaw in rats ($p < 0.01$). **b**) Ranges of E_{anion} recorded from LI neurons of naïve (Δ) and PNI (o) rats. Solid symbol = mean E_{anion} ± SEM. **c**) All classes of LI neurons (i.e. with phasic (P), single-spike (SS) and tonic (T) firing properties [19]) showed a shift in E_{anion} in response to PNI. Scale bar is 50 mV (y), 150 ms (x). **d**) Mean peak current measured in LI neurons from naïve (▲) and PNI (●) rats in response to applied GABA at various V_m. Horizontal standard error bars represent inter-neuron differences in recording pipette offset. Inset: Representative traces from one neuron. Scale bar is 0.6 nA (y), 1.0 s (x).

Figure 2: Switch from GlyR(receptor)-only to mixed $GABA_AR$- and GlyR-mediated miniature postsynaptic currents (mPSCs) following PNI in LI neurons. **a**) Raw traces of outward (left) and inward (right) miniature synaptic events from a naïve rat LI neuron. All outward mPSCs were abolished by strychnine, while all inward mPSCs (recorded in the presence of strychnine and bicuculline) were abolished by the GluR antagonist CNQX. HP - Holding Potential. Scale bar is 20 pA (y), 300 ms (x). **b**) Raw traces of inward (left) and outward (right) miniature synaptic events recorded from a PNI rat LI neuron. Unlike in naïve rats, both strychnine and bicuculline were required to abolish all outward mPSCs. Inward mPSCs remained completely sensitive to CNQX. Scale bar is 20 pA (y), 300 ms (x). **c**) left - Superimposed individual mPSCs recorded from PNI rat LI neurons. GlyR-only and $GABA_AR$-only and mixed $GABA_AR$/Glyr-mediated were clearly identifiable by their sensitivity to strychnine and/or bicuculline. Right - Averages of > 100 GlyR- and $GABA_AR$-mediated mPSCs recorded from a PNI rat LI neuron. Scale bar is 15 pA (y), 20 ms (x). **d**) Mean peak conductance of mPSCs recorded from naïve (N; n =

10 for GlyR; n = 5 for GluR) and PNI (P; n = 9 for GlyR; n = 8 for GluR) LI neurons. P(B) indicates GlyR-mediated mPSCs recorded in PNI rat LI neurons (n = 12) at 0 mV in the presence of bicuculline. **e)** Net charge carried by GlyR-mediated mPSCs in naïve rats (n = 6), by bicuculline-isolated GlyR-mediated mPSCs in PNI rats [PNI(Bic); n = 4], and by mixed $GABA_AR$/GlyR-mediated mPSCs in PNI rat LI neurons (PNI; n = 12). **f)** Cumulative probability plot illustrating the difference between the GlyR-only mPSC inter-event interval (I.E.I.) in naïve LI neurons and that of bicuculline-isolated GlyR-only mPSCs in PNI rat LI neurons [PNI(Bic)], both recorded at E_{anion}= 0 mV. The addition of the $GABA_AR$-mediated mPSCs (PNI) compensated the GlyR-only mPSC frequency decrease. Inset - No effect of PNI on the frequency of GluR-mediated mPSCs.

Figure 3: PNI-induced downregulation of KCC2 in SDH lamina I neurons ipsilateral to PNI led to GlyR/$GABA_AR$-mediated excitation. **a)** Brief GABA application (30 ms pressure puff) caused a tetrodotoxin (TTX) and bicuculline-sensitive rise in $[Ca^{2+}]_i$ in a LI neuron from a fura-2-am (Ca^{2+} indicator) loaded slices of a PNI rat. **b)** KCl application, but not GABA application (up to 250 ms-long pressure puffs) caused no change in $[Ca^{2+}]_i$ in a naïve rat LI neuron. In the presence of the KCC2-specific antagonist DIOA, GABA application did elicit a rise in $[Ca^{2+}]_i$ in a naïve rat LI neuron. Scale bar is 0.02 (y), 10 s (x). **c)** Percentage of LI neurons displaying a GABA-evoked increase in $[Ca^{2+}]_i$. The proportion was significantly higher in PNI rats ($\chi^2_{corrected}$ = 3.91) and in the presence of DIOA in naïve rats ($\chi^2_{corrected}$ = 4.43). **d)** Representative trace confirming that exogenous GABA could repeatedly elicit action potentials in a lamina I neuron. Upper scale bar is 5 mV (y), 200 ms (x). Lower scale bar is

30 mV (y), 4 s (x). Inset - response to a depolarizing pulse confirming this was a single-spike neuron (19). Scale bar is 20 mV (y), 300 ms (x). **e)** Similarly, focal stimuli (in the presence of glutamate receptor blockers) elicited bicuculline-sensitive monosynaptic depolarizing postsynaptic potentials thar could evoke action potentials in a lamina I neuron from PNI rats. Scale bar is 5 mV (y), 250 ms (x). Inset - response to a depolarizing pulse confirming this was a phasic neuron (19). Scale bar is 20 mV (y), 300 ms (x). **f)** Left - Immunoblotting revealed that KCC2 levels were decreased in the lumbar SDH lying ipsilateral (Ipsi), but not contralateral (Con), to the site of the PNI. Right - Average intensities (±SEM) of KCC2 protein (normalized to actin) measured from immunoblots (n = 4) as in left (Ipsi normalized to Con).

Figure 4: Selective blockade or knock-down of the postsynaptic KCC2 exporter in the SDH significantly reduced nociceptive threshold. **a)** Tactile nociceptive withdrawal threshold as a function of time after intrathecal injections of DIOA (n = 5) or vehicle (n = 3). **b)** Thermal nociceptive withdrawal latency as a function of time after intrathecal injections of DIOA (n = 3) or vehicle (n = 3). Upon withdrawal, the rats also often licked their paw indicating nociception. **c)** Spontaneous mPSCs recorded with a CsCl (cesium chloride) pipette (to clamp E_{anion} at 0mV) in a LI neuron in the presence and absence of DIOA. Scale bar is 20 pA (y), 300 ms (x). **d)** Cumulative probability plot (n = 4 neurons X 50 mPSCs) demonstrating that DIOA neither affected the peak conductance of synaptic events (p> 0.5), nor GABA-evoked responses (n = 5; p > 0.5, Inset) and therefore does not act on GlyRs nor $GABA_ARs$. G_{peak} = peak conductance. **e)** Local lumbar spinal (intrathecal) administration of a KCC2

antisense oligodeoxynucleotide (each 12 h) caused a significant decrease in the tactile nociceptive withdrawal threshold in naïve rats (n = 8), compared to those that received the scrambled oligodeoxynucleotide (n = 7). Inset, Decrease in spinal KCC2 protein levels (measured by immunoblots) following antisense (AS, 12h or 36h) or scrambled (S, 36h) oligodeoxynucleotide treatment. **f**) Lack of KCC2 immunoreactivity in dorsal root ganglia (DRG) in a naïve rat, compared to SDH. **g**) Electron micrograph illustrating the selective expression of KCC2 in SDH dendrites (D), but not synaptic boutons (B) (for quantitative details see Fig. 6). Arrows point to synapses. Scale bar is 0.2 µm.

Figure 5: Computer simulations of *in vivo* synaptic conditions confirmed that sensitization of Lamina I neurons occurred as a function of the shift in the E_{anion}. **a**) Left - Computer simulations using a model neuron (see Examples) demonstrate how PNI-induced changes to GlyR- and $GABA_AR$-mediated PSCs [PNI(GlyR+$GABA_AR$)] affect the output firing frequency of LI neurons as a function of GluR-mediated PSC frequency. Also shown is the result in LI neurons after PNI if only considering the effect of GlyR- mediated [PNI(GlyR-only)] or $GABA_AR$-mediated [PNI($GABA_AR$-only)] synaptic events. Right - Same data as shown in the left panel, but expressed in terms of firing frequency ratio, which was calculated as the quotient of a specific data set divided by the No Inhibition data set (i.e., a firing frequency ratio of one is equivalent to no inhibition). While the normally hyperpolarizing GlyR-mediated PSCs (mean E_{anion} = -72.8 mV in naïve rats) had a net inhibitory effect on the output firing frequency (f_{out}), depolarizing GlyR-mediated PSCs (mean E_{anion} = -49.0 mV in PNI rats), enhanced f_{out} beyond that predicted to result with no

inhibition, demonstrating a net excitatory effect. This excitatory effect was more prevalent when the $GABA_AR$ component was incorporated due to the increased charge carried by $GABA_AR$-mediated PSCs. **b**) Left - Effect of different values of E_{anion} (over the range observed in our study) on the firing frequency of a LI neuron after PNI. Right - Same data as left panel expressed in terms of firing frequency ratio (as above).

Figure 6: KCC2 exporter expression is restricted to dorsal horn neurons, not sensory fibres. Although the KCC2 levels are below detection by immunoblotting from DRG (Fig. 4f), we verified whether KCC2 could be preferentially shuttled away from cell bodies to central terminals of primary afferents. **a**) Electron micrograph illustrating the presence of KCC2 on dendrites (D) in lamina I of the dorsal horn. Membrane-delimited immunogold staining on the soma (S) of a lamina I neuron is also shown (arrowheads). In contrast, no KCC2 immunostaining was observed in any of the randomly selected synaptic profiles examined (n = 171). **b**) KCC2 immunoreactivity was also absent from central boutons (n = 42 randomly selected central boutons) of synaptic glomeruli in laminae I and II (type I: C_I; left; type II: C_{II}; right; arrows indicate excitatory synapses, D: dendrite) that unequivocally correspond to central terminals of primary afferents (A- and C- fibres [34,35]). Scale bars: a: 2 µm; b; 0.5 µm (left), 0.2 µm (right).

Figure 7: Effect of various treatments on anion (bicarbonate and chloride) reversal potential (E_{anion}) recorded from lamina I neurons of naïve and PNI rats.

Figure 8: Intrathecal administration of the receptor tyrosine kinase inhibitor K-252a (6 nM) resulted in an increase in the threshold for tactile nociceptive withdrawal.

Figure 9: DNA (SEQ ID NO: 1) and polypeptide (SEQ ID NO: 2) sequences of human KCC2.

Figure 10: DNA (SEQ ID NO: 3) and polypeptide (SEQ ID NO: 4) sequences of mouse KCC2.

Figure 11: DNA (SEQ ID NO: 5) and polypeptide (SEQ ID NO: 6) sequences of rat KCC2.

Figure 12: Comparison of the anion (chloride and bicarbonate) reversal potential (E_{anion}) measured from lamina I neurons in slices, taken from naïve rats, perfused with BDNF, NGF or regular artificial cerebrospinal solution (ACSF; "control" in Figure). PNI - peripheral nerve injury.

Figure 13: Comparison of E_{anion} measured in slices containing lamina I neurons taken from PNI rats treated, by bath application, with an antibody directed against TrkB (P/TrkBIgG), H-89 (P/H89), K-252a (P/K252a) and KN-93 (P/KN93). PNI - peripheral nerve injury.

Figure 14: Comparison between the nociceptive threshold for tactile stimulation of rats treated with an adenovirus transducing BDNF (■) and rats treated with an adenovirus transducing the green fluorescent protein (O).

Figure 15: Comparison between the nociceptive threshold for tactile stimulation of rats treated with human recombinant

NGF (10 µg/day x 6 days) (■) and rats treated with saline vehicle(O).

Figure 16: Comparison between the nociceptive threshold for tactile stimulation of rats treated with the neutralizing anti-TrkB antibody (anti-TrkB-IgG 12 µg/2 hrs x 3) (■) and rats treated with vehicle only (O).

Figure 17: Comparison between the nociceptive threshold for mechanical stimulation of rats treated with the PKA inhibitor H-89 (380 nmol) (■) and rats treated with vehicle only (O).

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a novel mechanism of disinhibition following peripheral nerve injury. It involves a transsynaptic disruption of anion homeostasis in neurons of lamina I of the superficial dorsal horn (SDH), one of the main spinal nociceptive output pathways (7). The resulting shift in the transmembrane anion gradient is shown herein to cause normally inhibitory anionic synaptic currents to be excitatory, substantially driving up the net excitability of lamina I neurons. As shown herein, peripheral nerve injury is sensed by transmission of a signal transynaptically resulting in an increase in intracellular chloride levels in central nervous system (CNS) neurons. Further, the studies described herein demonstrate that decreasing CNS neuronal chloride levels can reverse this phenomenon, as shown via local blockade or knock-down of the spinal KCC2 exporter in intact rats which markedly reduced nociceptive threshold, confirming that the reported disruption of anion homeostasis in lamina I neurons was sufficient to cause neuropathic pain.

Therefore, the studies described herein have investigated the mechanism of pain sensation via the study of downstream events following peripheral nerve injury. As such, it is shown herein that such events are transmitted transsynaptically (e.g. by a peripheral nervous system (PNS cell or a sensory fiber) to central nervous system (CNS) neurons, in an embodiment, to spinal cord neurons. Further studies herein demonstrate that transmission of the nociceptive signal and sensation of pain is ultimately effected by a modulation of intracellular chloride level (e.g. modulated by a chloride transporter such as the potassium-chloride cotransporter KCC2) in a CNS tissue. KCC2 (see (37) for a review) is a potassium-chloride cotransporter which has been identified in rat, mouse and human (for human KCC2 see for example US Patent application Serial No. 20030027983 of Mount et al.; published February 6, 2003). Studies of homozygous and heterozygous disruptions of the KCC2 gene in mouse revealed a seizure phenotype, suggesting a possible role for KCC2 in epilepsy (38). The precise role of KCC2 in CNS function is not yet completely understood.

Applicants demonstrate herein a correlation between the intracellular chloride level (e.g. by virtue of the activity/expression of a chloride transporter such as KCC2) in a CNS cell or tissue, and the sensation of pain. As shown in the examples below, peripheral nerve injury (PNI) results in the hyperexcitation or sensitization of CNS neurons, e.g. of the spinal cord, e.g. lamina I (LI) neurons of the superficial dorsal horn (SDH). Such hyperexcitability occurs transsynaptically (i.e. downstream from the injured peripheral neuron), a phenomenon which has not been described prior to applicants' studies herein. Such hyperexcitability results in a reduction of the nociceptive threshold.

As shown herein, the hyperexcitability noted above correlates with an increase in intracellular chloride levels (e.g. modulation [e.g. decrease] in chloride transporter [such as KCC2] activity and/or expression) in the SDH. The role of KCC2 in this regard was confirmed via administration of the KCC2 blocker DIOA or a KCC2 antisense oligonucleotide to spinal tissue, both resulting in a rapid decrease in the threshold for pain sensitivity. Therefore, a reduction in KCC2 activity and/or expression, if it results in increased CNS neuronal chloride levels, may result in a decrease in the threshold for pain sensitivity, and, conversely, an increase or induction of KCC2 activity and/or expression, if it results in a decrease in CNS neuronal chloride, may result in an increase in the threshold for pain sensitivity thus providing for prevention and treatment of pain. On the other hand, it has been reported that under certain pathophysiological conditions, e.g. where $[K^+]_o$ is elevated, KCC2 may accumulate Cl^- in neurons, thereby enhancing neuronal excitability (42). Under such conditions, it is envisioned that KCC2 would have the opposite effect on CNS neuronal chloride, and thus result in an increase in CNS neuronal chloride and in turn decreased nociceptive threshold and increased pain sensation. As such, modulation of the activity and/or expression of KCC2 may, depending on the directionality of the flux of chloride ion, contribute to or alleviate a pain sensation.

Therefore, in a first aspect, the invention relates to methods and materials for the treatment of pain, based on the modulation of CNS intracellular chloride level and further the modulation of the activity and/or expression of a chloride transporter, e.g. the KCC2 potassium-chloride cotransporter. As used herein, a "chloride transporter" is defined as a polypeptide/protein or complex thereof

associated with the cell membrane that is able to effect the passage of chloride anions across the cell membrane. "Export(er)" refers to a net passage from the inside to the outside of the cell, and "import(er)" refers to a net passage from the outside to the inside of the cell.

Therefore, in an embodiment, the present invention relates to methods for treating pain by decreasing the intracellular chloride level in a cell, e.g. a CNS neural cell. In a further embodiment, modulators of a chloride transporter (e.g. KCC2) can be used to decrease intracellular chloride levels. In an embodiment, the invention relates to the application, systemic or local, of compounds or drugs that decrease the intracellular level of chloride in a CNS neural cell as a means to attenuate pain. In order achieve this result, the above-mentioned compounds or drugs may modulate the function or expression of the chloride transporter (e.g. KCC2 cotransporter) in CNS neurons. In yet a further embodiment, the compounds or drugs may increase the expression or activity of the chloride transporter or KCC2.

In an embodiment, the CNS neural cell in which the intracellular chloride levels are being modulated can be located in the superficial dorsal horn or the spinal cord. In addition, the cell may also be transsynaptic to a peripheral nerve cell or sensory fiber from which a signal for pain originates.

In an embodiment, the invention also relates to the treatment of acute and chronic pain, more specifically to the treatment of neuropathic pain. "Neuropathic pain", as used herein, refers to chronic pain associated with nerve injury (e.g. following crush, transection or compression of nerves or following nerve degeneration resulting from disease). In an embodiment, neuropathic pain is associated with a nerve or tract injury. In a further embodiment, the neuropathic pain

is associated with visceral and/or somatic pain. The invention further relates to decreasing CNS neuronal chloride levels (e.g. via modulation of chloride transporter [such as KCC2] activity and/or expression) to reduce nociception. "Nociception" as used herein refers to the sensory component of pain. Pain may be the result of various stimuli, including but not limited to pressure, injury, thermal stimuli or chemical (e.g. ionic) stimuli. In embodiments, the pain may be associated with many conditions such as chronic inflammatory pain, pain associated with arthritis, fibromyalgia, back pain, cancer-associated pain, pain associated with digestive disease, pain associated with Crohn's disease, pain associated with autoimmune disease, pain associated with endocrine disease, pain associated with diabetic neuropathy, phantom limb pain, spontaneous pain, chronic post-surgical pain, chronic temporomandibular pain, causalgia, post-herpetic neuralgia, AIDS-related pain, complex regional pain syndromes type I and II, trigeminal neuralgia, chronic back pain, pain associated with spinal cord injury and/or recurrent acute pain. The invention also relates to methods of diagnosis and prognostication to assess pain associated with CNS dysfunction. In an embodiment, such diagnosis/prognostication may be performed prior to the method of treatment described herein, or during a treatment regimen, to further characterize the nature of the pain or its progression, and thus provide information which may be used e.g. to select a course of treatment for such pain in accordance with the results obtained from such diagnosis/prognostication. As used herein, "pain associated with CNS dysfunction" relates to a pain sensation that is caused by an alteration in ion (e.g. anion) homeostasis in a CNS tissue. In an embodiment, the anion is a chloride ion. In a further embodiment, the alteration is an increase in an

intracellular chloride level in a CNS cell. In yet another embodiment, the activity or expression of a chloride transporter may be modulated (e.g. KCC2 activity or expression may be modulated [e.g. decreased]) when a subject experiences pain associated with a CNS dysfunction.

"KCC2" as used herein refers to a particular type of potassium-chloride cotransporter expressed in neurons. In embodiments, KCC2 comprises the sequence of the polypeptide of SEQ ID NOs: 2 (human KCC2; see also Figure 9), 4 (mouse KCC2; see also Figure 10) or 6 (rat KCC2; see also Figure 11), fragments thereof or sequences substantially identical thereto. In further embodiments, KCC2 is encoded by the nucleic acid sequences capable of encoding the polypeptides of SEQ ID NO: 2, 4 or 6, or fragments thereof or sequences substantially identical thereto or related by hybridization criteria (see below). In further embodiments, such nucleic acid sequences comprise of SEQ ID NO: 1 (human KCC2 DNA; see also Figure 9), 3 (mouse KCC2 DNA; see also Figure 10) or 5 (rat KCC2 DNA; see also Figure 11), fragments thereof or sequences substantially identical thereto or related by hybridization criteria (see below).

"Chloride transport(er) activity" as used herein refers to the transport of chloride, across the cell membrane. Such transport activity may be measured by direct or indirect means using various methods known in the art, examples of which are described herein. "KCC2 activity" as used herein refers to any detectable phenotype associated with KCC2. In an embodiment, KCC2 activity includes, but is not limited to potassium transport, chloride transport, which may, for example, be determined by assessing levels (either directly or indirectly) of potassium and/or chloride inside and/or outside the cell using, for example, reversal potential measurements with patch clamping methods,

chloride/potassium sensitive dyes (see for example Haugland, R.P., *Handbook of Fluorescent Probes and Research Products*, ninth ed., 2002, Molecular Probes, Inc., Eugene, OR, USA) electrodes, etc. In addition, KCC2 activity may also affect the neural cell's anion reversal potential (E_{anion}). The anion reversal potential may be determined, for example, by using gramicidin-perforated patch clamp recording.

"Chloride transporter expression" (e.g. KCC2 expression) relates both to production of a chloride transporter transcript (e.g. a KCC2 transcript) or a chloride transporter polypeptide or protein (e.g. a KCC2 polypeptide or protein). Chloride transporter expression (e.g. KCC2 expression) may therefore, in embodiments, be determined by assessing protein levels directly (e.g., by immunocytochemistry and/or western analysis) or a level of a chloride transporter-encoding nucleic acid (e.g. chloride transporter-encoding nucleic acid such as chloride transporter mRNA levels) that may be determined by using, for example, methods such as reverse-transcriptase polymerase chain reaction [RT-PCR] methods, micro-array-based methods or by Northern analysis).

Compounds capable of decreasing intracellular chloride level in a CNS neural cell may, for example, modulate chloride transporter activity and expression (e.g. KCC2 activity and expression). In an embodiment, the chloride transporter activity or expression (e.g. KCC2 activity or expression) may be increased. In an embodiment, these compounds can be administered in a way such that they contact a CNS tissue or a CNS cell. The compounds that can be used include, but are not limited to, those which directly or indirectly modify the activity of the protein and those which modulate the production and/or stability of the protein (e.g. at the level of transcription, translation, maturation,

post-translationnal modification, phosphorylation and degradation).

One class of such compounds are those that act via modulation of phosphorylation of one or more sites on KCC2. Upon cloning KCC2 (20), it has been reported that KCC2 does not contain consensus phosphorylation sites for PKA, yet does contain five for PKC (Thr^{34}, Ser^{728}, Thr^{787}, Ser^{940} & Ser^{1034}). One consensus site was identified for tyrosine protein phosphorylation (Tyr^{1081}) in the carboxyl-terminal. This tyrosine kinase consensus phosphorylation site is not present in the KCC1 or KCC4 isoforms, yet it is conserved in the KCC3 protein (21). As such, compounds capable of upregulating or increasing KCC2 activity include, but are not limited to, protein kinases inhibitors (e.g. N-ethylmaleimide (23-25), staurosporine (29), and receptor tyrosine kinase inhibitors such as K-252a); antibodies or antibody fragments generated against certain kinases or kinase phosphorylation sites on KCC2, or compounds which interfere more directly (e.g. oligopeptides capable of competing with phosphorylation sites on KCC2) or less directly (e.g. compounds which modulate kinase activity and/or expression) with KCC2 phosphorylation. In an embodiment, such a compound may act at the level of phosphorylation-mediated signaling pathways and ultimately affect KCC2 phosphorylation. In another embodiment, TrkB may be modulated to affect KCC2 phosphorylation and ultimately modulate KCC2 activity. Thus, In an embodiment, compounds that inhibit TrkB activity may, for example, be used in this regard. Such compounds may include, but are not limited to, K-252a (commercially available from Calbiochem) or a neutralizing antibody against TrkB (anti-TrkB antibody [e.g. IgG])(commercially available from BD Transduction Laboratories). In yet another embodiment, modulation, e.g. inhibition, of cyclic AMP-dependant kinase or PKA may be

useful in modulating KCC2 phosphorylation and ultimately be used in the treatment or prevention of pain. For example, the PKA inhibitor H-89 (commercially available from EMD Biosciences) may be used in this regard. In a further embodiment, modulation, e.g. inhibition, of calmodulin-dependant kinase (CAM kinase, e.g. II and IV) may alleviate or prevent pain in a subject by modulating KCC2 activity, e.g. phosphorylation. Compounds capable of inhibiting such a kinase include, but are not limited to, KN-93 (commercially available from EMD Biosciences). In yet another embodiment, modulators, e.g.inhibitors, of other members of the TrkB pathway, e.g. phosphatidylinositol-specific phospholipase C or phosphatidylcholine-specific phospholipase C, e.g. phospholipase C gamma (PLCγ), may be used to decrease intracellular chloride levels in a CNS neural cell. Such compounds include, but are not limited to, tricyclodecan-9-yl-xanthogenate, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine, neomycin sulfate, spermine tetrahydrochloride, 1-[6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione, or 1-[6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione.

Further, modulation of KCC2 expression may also arise from modulation (e.g. mediated by phosphorylation) of transcription factors which regulate KCC2 expression.

In a further aspect, the invention provides a method for treating pain or preventing/decreasing nociception in a subject or animal, comprising modulating, in embodiments reducing or decreasing, intracellular chloride levels in a CNS neuron or tissue. In an embodiment, such decrease in intracellular chloride levels is achieved by modulating, e.g. decreasing, activity or expression of a chloride transporter (e.g. KCC2) in a CNS neuron or tissue of the subject. In a

further embodiment, the subject is a vertebrate. In another embodiment, the subject is a mammal, in a yet further embodiment, a human. In an embodiment, the CNS tissue is spinal cord tissue and the neural cell is a spinal cord neural cell.

Accordingly, the invention therefore provides methods of treating pain comprising administering a compound capable of modulating, in an embodiment, decreasing or reducing intracellular chloride levels in CNS tissue (e.g. a CNS neural cell) in a subject. In an embodiment, the modulation, e.g. increase, in chloride transporter (e.g. KCC2) activity and/or expression effects the decrease in intracellular chloride level in the subject. In an embodiment, the CNS tissue is spinal cord tissue and the neural cell is a spinal cord neural cell.

In an embodiment, KCC2 comprises an amino acid sequence substantially identical to a sequence set forth in SEQ ID NO: 2, 4, 6 or a fragment thereof. In another embodiment, KCC2 may be encoded by a nucleic acid substantially identical to a nucleotide sequence capable of encoding SEQ ID NO: 2, 4, 6 or a fragment thereof, such as a sequence substantially identical to the sequence set forth in SEQ ID NO: 1, 3, 5 or a fragment thereof.

As noted above, a homolog, variant and/or fragment of a KCC2 which retains activity may also be used in the methods of the invention. Homologs include protein sequences which are substantially identical to the amino acid sequence of a KCC2, sharing significant structural and functional homology with a KCC2. Variants include, but are not limited to, proteins or peptides which differ from a KCC2 by any modifications, and/or amino acid substitutions, deletions or additions. Modifications can occur anywhere including the polypeptide backbone, (i.e. the amino acid sequence), the

amino acid side chains and the amino or carboxy termini. Such substitutions, deletions or additions may involve one or more amino acids. Fragments include a fragment or a portion of a KCC2 or a fragment or a portion of a homolog or variant of a KCC2.

With regard to increasing or upregulating expression of KCC2 in a cell, various methods of introducing KCC2-encoding nucleic acids into the cell may be used, examples of which are described below. Methods such as the gene therapy methods discussed below may be used in this regard. Examples of KCC2-encoding nucleic acids include nucleic acids capable of encoding a polypeptide of SEQ ID NO: 2, 4 or 6 (e.g. the nucleic acids of SEQ ID NO: 1, 3 and 5), or nucleic acids substantially identical thereto. The method may also comprise administering to an area or neural tissue, e.g. CNS tissue, a cell comprising such a KCC2-encoding nucleic acid, via for example transplantation or introduction of a neural cell or precursor thereto (e.g. a stem cell) comprising such a KCC2-encoding nucleic acid. Further, the method may entail administering to the subject a compound capable of modulating, e.g. unpregulating or increasing, expression of a KCC2. Such a compound may for example be identified and characterized by the screening methods described below. Such a compound may further be provided as a composition comprising the compound and a pharmaceutically acceptable carrier. In an embodiment, the composition is formulated for or adapted for administration to the CNS. Such a compound or composition may be provided in a commercial package together with instructions for its use.

"Homology" and "homologous" refers to sequence similarity between two peptides or two nucleic acid molecules. Homology can be determined by comparing each position in the aligned sequences. A degree of homology

between nucleic acid or between amino acid sequences is a function of the number of identical or matching nucleotides or amino acids at positions shared by the sequences. As the term is used herein, a nucleic acid sequence is "homologous" to another sequence if the two sequences are substantially identical and the functional activity of the sequences is conserved (as used herein, the term "homologous" does not infer evolutionary relatedness). Two nucleic acid sequences are considered substantially identical if, when optimally aligned (with gaps permitted), they share at least about 50% sequence similarity or identity, or if the sequences share defined functional motifs. In alternative embodiments, sequence similarity in optimally aligned substantially identical sequences may be at least 60%, 70%, 75%, 80%, 85%, 90% or 95%. As used herein, a given percentage of homology between sequences denotes the degree of sequence identity in optimally aligned sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than about 25 % identity, with any of SEQ ID NO: 1 to 6.

Substantially complementary nucleic acids are nucleic acids in which the "complement" of one molecule is substantially identical to the other molecule. Optimal alignment of sequences for comparisons of identity may be conducted using a variety of algorithms, such as the local homology algorithm of Smith and Waterman, 1981, *Adv. Appl. Math* 2: 482, the homology alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85: 2444, and the computerised implementations of these algorithms (such as GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI, U.S.A.). Sequence identity may also be

determined using the BLAST algorithm, described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-10 (using the published default settings). Software for performing BLAST analysis may be available through the National Center for Biotechnology Information (through the internet at http://www.ncbi.nlm.nih.gov/). The BLAST algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold. Initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when the following parameters are met: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program may use as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992, *Proc. Natl. Acad. Sci. USA* 89: 10915-10919) alignments (B) of 50, expectation (E) of 10 (or 1 or 0.1 or 0.01 or 0.001 or 0.0001), M=5, N=4, and a comparison of both strands. One measure of the statistical similarity between two sequences using the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. In alternative embodiments of the invention, nucleotide or amino

acid sequences are considered substantially identical if the smallest sum probability in a comparison of the test sequences is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

An alternative indication that two nucleic acid sequences are substantially complementary is that the two sequences hybridize to each other under moderately stringent, or preferably stringent, conditions. Hybridization to filter-bound sequences under moderately stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C (see Ausubel, *et al.* (eds), 1989, *Current Protocols in Molecular Biology*, Vol. 1, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Alternatively, hybridization to filter-bound sequences under stringent conditions may, for example, be performed in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.1 x SSC/0.1% SDS at 68°C (see Ausubel, *et al.* (eds), 1989, *supra*). Hybridization conditions may be modified in accordance with known methods depending on the sequence of interest (see Tijssen, 1993, *Laboratory Techniques in Biochemistry and Molecular Biology -- Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York). Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH.

According to a further aspect, the invention also provides a method for decreasing nociception in a subject. In an embodiment, this method comprises modulating, e.g. decreasing, intracellular chloride levels in a cell, e.g. a

CNS cell, in a subject. In a further embodiment, the method also comprises modulating, e.g. increasing, chloride transporter activity or expression, e.g. KCC2 activity or expression. In yet another embodiment, the method also comprises contacting the CNS neural cell with a compound capable of modulating chloride transporter activity. Such compounds include, but are not limited to a TrkB inhibitor (such as K-252a or anti-TrkB antibody), a PKA inhibitor (such as H-89) or a CAM kinase inhibitor (such as KN-93).

The invention further provides a composition for the prevention and/or treatment of pain comprising a compound capable of modulating, e.g. decreasing, intracellular chloride levels in admixture with a pharmaceutically acceptable carrier. In an embodiment, such composition may modulate, e.g. increase or upregulate, chloride transporter activity, e.g. KCC2, activity and/or expression. In an embodiment, such a composition is suitable for or adapted for administration to a CNS neural cell or tissue, such as spinal cord tissue or cell. In yet a further embodiment, such a composition may be an inducer of KCC2 expression or activity. As used herein, an "inducer" is a compound that upregulates or enhances directly or indirectly the expression of the KCC2 gene, stability of the KCC2 mRNA, translation of the KCC2 mRNA, maturation of the KCC2 polypeptide, transport, e.g. recycling, of the KCC2 polypeptide to the cell membrane, or transporter activity of the KCC2 polypeptide. In an embodiment, the "inducer" can also down-regulate or inhibit KCC2 inhibitors.

The invention further provides a use of the above-mentioned composition or the above-mentioned compound, capable of modulating, e.g. decreasing, intracellular chloride levels for the treatment or prevention of pain. The invention also provides a use of the above-mentioned

composition or the above-mentioned compound, capable of modulating, e.g. decreasing, intracellular chloride levels for the preparation of a medicament for treatment or prevention of pain. In an embodiment, the compound or composition modulates, e.g. increases or upregulates, chloride transporter (e.g. KCC2) activity and/or expression. In yet another embodiment, the compound or composition may comprise a TrkB inhibitor (such as K-252a or anti-TrkB antibody), a PKA inhibitor (such as H-89) or a CAM kinase inhibitor (such as KN-93). In yet another embodiment, the medicament may be formulated for administration to a CNS tissue, e.g. CNS cell, of a subject. Further, the compound may be, for example, an inducer of KCC2 expression or activity.

The invention further provides commercial packages comprising a compound capable of modulating, e.g. decreasing, intracellular chloride levels or the above-described composition together with instructions for its use in the treatment or prevention of pain. In an embodiment, the compound may modulate, e.g. increase or upregulate, chloride transporter or KCC2 activity and/or expression.

In various embodiments, a compound capable of modulating, e.g. decreasing, intracellular chloride levels in a CNS cell may be used therapeutically in formulations or medicaments to treat pain. The compound may, for example, modulate, e.g. increase or upregulate chloride transporter (e.g. KCC2) activity and/or expression. The invention also provides corresponding methods of medical treatment, in which a therapeutic dose of a compound capable of modulating, in an embodiment decreasing, intracellular chloride levels, is administered in a pharmacologically acceptable formulation. Accordingly, the invention also provides therapeutic compositions comprising a compound capable of modulating, in

an embodiment decreasing intracellular chloride levels, and a pharmacologically acceptable excipient or carrier. The therapeutic composition may be soluble in an aqueous solution at a physiologically acceptable pH.

In an embodiment, a compound of the invention is administered such that it comes into contact with a CNS tissue or a CNS neuron. As used herein, the "central nervous system" or CNS is the portion of the nervous system comprising the brain and the spinal cord (e.g. in the lumbar region). By contrast, the "peripheral nervous system" or PNS is the portion of the nervous system other than the brain and the spinal cord. In an embodiment, the CNS tissue is the superficial dorsal horn, in a further embodiment, a lamina I neuron. As such, in embodiments a compound of the invention can be administered to treat CNS cells *in vivo* via direct intracranial or intrathecal injection or injection into the cerebrospinal fluid. Alternatively, the compound can be administered systemically (e.g. intravenously, or orally) in a form capable of crossing the blood brain barrier and entering the CNS. "Neural" and "neuronal" are used herein interchangeably and both relate to neurons and the nervous system.

The invention also provides pharmaceutical compositions (medicaments) comprising a compound capable of modulating, in an embodiment decreasing intracellular chloride levels in a CNS cell. In an embodiment, such compositions include the compound, in a therapeutically or prophylactically effective amount sufficient to treat or attenuate pain, and a pharmaceutically acceptable carrier. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction of pain. A therapeutically effective amount of a compound

capable of modulating, in an embodiment decreasing, intracellular chloride levels in a CNS cell, may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the compound to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting onset of pain or increases in the severity of pain. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions.

As used herein "pharmaceutically acceptable carrier" or "excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Alternatively, the carrier can be suitable for intravenous, intraperitoneal, intramuscular, intracranial, intrathecal, sublingual or oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any

conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the compound capable of modulating, in an embodiment increasing or upregulating, KCC2 activity and/or expression, can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic

copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g. a compound capable of modulating, in an embodiment decreasing, intracellular chloride levels in a CNS cell) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. In accordance with an alternative aspect of the invention, a compound capable of modulating, in an embodiment decreasing, intracellular chloride levels in a CNS cell, may be formulated with one or more additional compounds that enhance its solubility.

In accordance with another aspect of the invention, therapeutic compositions of the present invention, comprising a compound capable of modulating, in an embodiment decreasing, intracellular chloride levels in a CNS cell, may be provided in containers or commercial packages which further comprise instructions for their use for the treatment of pain.

Given that a decreased intracellular chloride level in a cell is associated with a modulation, e.g. an increase, in level/activity of chloride transporter (KCC2),

which further correlates with a decrease in pain sensation as described herein, a further aspect of the present invention is the treatment of pain by administering to a subject (e.g. to CNS tissue) a nucleic acid molecule encoding a KCC2, or a variant or fragment thereof which retains KCC2 activity. Suitable methods of administration include gene therapy methods.

A nucleic acid of the invention may be delivered to cells *in vivo* using methods such as direct injection of DNA, receptor-mediated DNA uptake, viral-mediated transfection or non-viral transfection and lipid based transfection, all of which may involve the use of gene therapy vectors. Direct injection has been used to introduce naked DNA into cells *in vivo* (see e.g., Acsadi et al. (1991) Nature 332:815-818; Wolff et al. (1990) Science 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* may be used. Such an apparatus may be commercially available (e.g., from BioRad). Naked DNA may also be introduced into cells by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C. H. (1988) J. Biol. Chem. 263:14621; Wilson el al. (1992) J. Biol. Chem. 267:963-967; and U.S. Pat. No. 5,166,320). Binding of the DNA-ligand complex to the receptor may facilitate uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which disrupt endosomes, thereby releasing material into the cytoplasm, may be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel el al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; Cristiano et al. (1993) Proc. Natl. Acad. Sci. USA 90:2122-2126). Defective retroviruses are well characterized for use as gene therapy vectors (for a review see Miller, A. D. (1990) Blood

76:271). Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include .psi.Crip, .psi.Cre, .psi.2 and .psi.Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

For use as a gene therapy vector, the genome of an adenovirus may be manipulated so that it encodes and expresses a polypeptide compound of the invention, but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155. Suitable

adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*), endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), hepatocytes (Herz and Gerard (1993) Proç. Natl. Acad. Sci. USA 90:2812-2816) and muscle cells (Quantin el al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584).

Adeno-associated virus (AAV) may be used as a gene therapy vector for delivery of DNA for gene therapy purposes. AAV is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). AAV may be used to integrate DNA into non-dividing cells (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 may be used to introduce DNA into cells (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790). Lentiviral gene therapy vectors may also be adapted for use in the invention.

General methods for gene therapy are known in the art. See for example, U.S. Pat. No. 5,399,346 by Anderson *et*

al. A biocompatible capsule for delivering genetic material is described in PCT Publication WO 95/05452 by Baetge *et al.* Methods of gene transfer into hematopoietic cells have also previously been reported (see Clapp, D. W., et al., Blood 78: 1132-1139 (1991); Anderson, Science 288:627-9 (2000); and Cavazzana-Calvo *et al.*, Science 288:669-72 (2000)). The invention further relates to transplantation methods, to introduce into a subject a cell comprising a nucleic acid capable of encoding a KCC2, or to introduce into a subject a cell which has been treated *in vitro* or *ex vivo* with a compound capable of decreasing intracellular chloride levels (e.g. by culturing the cell in an appropriate medium comprising the compound). In an embodiment, such a cell is a neural cell or a precursor thereof, e.g. a stem cell capable of developing/differentiating into a neural cell (neuron progenitor cell). Methods relating to neural stem cell isolation, proliferation, characterization and/or transplantation are described in for example US patents 5,851,832; 5,968,829; 5,411,883; 5,750,376; 6,040,180; 5,753,506 and 6,001,654. The nucleic acid may be present in a vector as described above, the vector being introduced into the cell *in vitro*, using for example the methods described above. In an embodiment, the cell is autologous, and is obtained from the subject. In embodiments, the cell is allogeneic or xenogeneic.

Given the correlation between intracellular chloride levels in a CNS cell and pain, compounds which are capable of modulating, e.g. decreasing, intracellular chloride levels in a CNS cell can be used for the prevention and treatment of pain. In an embodiment, compounds that modulate, e.g. increase or upregulate, chloride transporter, such as KCC2, activity/expression can be used for decreasing intracellular chloride levels and ultimately prevent or treat

pain. Therefore, the invention further relates to screening methods for the identification and characterization of compounds capable of modulating intracellular chloride levels and/or chloride transporter activity and/or expression. Therefore, the invention further provides a method of determining whether a candidate compound is capable of modulating intracellular chloride levels in a cell, and in turn is useful for the prevention and treatment of pain. In an embodiment, the method comprises contacting a CNS-derived cell with said candidate compound and determining whether the intracellular chloride level has decreased in the presence of the test compound. A decrease in intracellular chloride level is indicative that the test compound may be used for the treatment or the prevention of pain. As used herein, a "CNS-derived cell" is a cell isolated or derived from a CNS tissue, and in embodiments includes both primary neuronal cultures, immortalized neuronal cell lines, as well as accepted *in vitro* neuronal model systems (e.g. cells differentiated into neurons *in vitro*). In an embodiment, the above-mentioned cell possesses a chloride transporter or KCC2 activity. In yet a further embodiment, the cell endogenously expresses a chloride transporter (e.g. KCC2). In a further embodiment the above-mentioned cell has been genetically engineered to express a chloride transporter gene or a KCC2 gene. In an embodiment, the cell may be an appropriate host cell comprising an exogenously introduced source of a chloride transporter, such as KCC2. Such a host cell may be prepared by the introduction of nucleic acid sequences encoding a chloride transporter or KCC2 into the host cell and providing conditions for the expression of such nucleic acid. In an embodiment, such a nucleic acid is DNA. Such host cells may be eukaryotic, such as amphibian or mammalian cells. In an embodiment, such host cells are human.

The invention also provides another method for the identification or characterization of compounds useful for the treatment and prevention of pain. In an embodiment, the method comprises contacting a CNS-derived cell with the candidate compound and determining whether chloride transporter activity has been modulated in the presence of the test compound. A modulation, e.g. increase, in chloride transporter activity is indicative that the test compound may be used for the treatment or the prevention of pain. In an embodiment, the chloride transporter is KCC2. KCC2 activity may be determined, for example, by measuring potassium transport, chloride transport, intracellular chloride levels and anion reversal potential.

The above-mentioned methods may be employed either with a single test compound or a plurality or library (e.g. a combinatorial library) of test compounds. In the latter case, synergistic effects provided by combinations of compounds may also be identified and characterized. The above-mentioned compounds may be used for prevention and/or treatment of pain, or may be used as lead compounds for the development and testing of additional compounds having improved specificity, efficacy and/or pharmacological (e.g. pharmacokinetic) properties. In an embodiment the compound may be a prodrug which is altered into its active form at the appropriate site of action, e.g. in CNS tissue (e.g. in the spinal cord). In certain embodiments, one or a plurality of the steps of the screening/testing methods of the invention may be automated.

As noted above, the invention further relates to methods for the identification and characterization of compounds capable of modulating, in an embodiment increasing, chloride transporter , e.g. KCC2, gene expression. Such a method may comprise assaying chloride transporter, e.g. KCC2,

gene expression in the presence versus the absence of a test compound. Such gene expression may be measured by detection of the corresponding RNA or protein, or via the use of a suitable reporter construct comprising a transcriptional regulatory element(s) normally associated with such chloride transporter or KCC2 gene, operably-linked to a reporter gene. A first nucleic acid sequence may "operably-linked" with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably-linked to a coding sequence if the promoter affects the transcription or expression of the coding sequences. Generally, operably-linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in reading frame. However, since, for example, enhancers generally function when separated from the promoters by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably-linked but not contiguous. "Transcriptional regulatory element" is a generic term that refers to DNA sequences, such as initiation and termination signals, enhancers, and promoters, splicing signals, polyadenylation signals which induce or control transcription of protein coding sequences with which they are operably-linked. The expression of such a reporter gene may be measured on the transcriptional or translational level, e.g. by the amount of RNA or protein produced. RNA may be detected by for example Northern analysis or by the reverse transcriptase-polymerase chain reaction (RT-PCR) method (see for example Sambrook *et al* (1989) Molecular Cloning: A Laboratory Manual (second edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA). Protein levels may be detected either directly using affinity reagents (e.g. an antibody or fragment thereof [for methods,

see for example Harlow, E. and Lane, D (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY]; a ligand which binds the protein) or by other properties (e.g. fluorescence in the case of green fluorescent protein) or by measurement of the protein's activity, which may entail enzymatic activity to produce a detectable product (e.g. with altered spectroscopic properties) or a detectable phenotype (e.g. alterations in cell growth). Suitable reporter genes include but are not limited to chloramphenicol acetyltransferase, beta-D galactosidase, luciferase, or green fluorescent protein.

In an embodiment, a candidate compound may further be assayed to determine if it is capable of modulating a chloride transporter-mediated process (e.g. KCC2-mediated process) or chloride transporter activity (e.g. KCC2 activity). In an embodiment, such chloride transporter-mediated process is ion transport, e.g. potassium or chloride transport, as determined by for example by assessing potassium and/or chloride levels (e.g. intracellularly) or by measuring anion reversal potential (electrophysiologically), membrane potential, for example as described in the examples below.

The invention also relates to the diagnosis and prognostication of pain. In an embodiment, the pain is caused by an alteration in ion, e.g. anion or chloride, homeostasis in the nervous system, e.g. central nervous system, of a subject. Without wishing to being bound to any particular theory, a reduced capacity of potassium and chloride export from neurons in the central nervous system (CNS) may lead to persistent neuronal hyperexcitability and ultimately pain.

The invention thus provides a method for diagnosing or prognosticating pain associated with CNS dysfunction. As used herein, "CNS dysfunction" is an alteration in neuronal

ionic homeostasis in the CNS. In an embodiment, the pain associated with such CNS dysfunction is neuropathic pain. In an embodiment, the method comprises determining an intracellular chloride level in a CNS neural cell and comparing the chloride level to a corresponding control level. In this particular method, an increase in the test level relative to a control level is an indication that the subject is experiencing pain associated with CNS dysfunction. In an embodiment, the method may comprise determining whether CNS chloride transporter activity or expression (e.g. KCC2 activity or expression) is modulated, e.g. upregulated or increased, relative to a control activity or expression. In yet another embodiment, the control chloride level can be selected from an established standard, a corresponding chloride level determined in the subject at an earlier time; a corresponding chloride level determined in said subject when the subject is experiencing less pain (relative to the current sensation of pain noted above) or substantially no pain; or a corresponding chloride level determined in a control subject experiencing less pain (relative to the current sensation of pain in the test subject noted above) or substantially no pain. In an embodiment, a subject or control subject experiencing less pain or substantially no pain presents no evident lesions to his central or peripheral nervous system (e.g. neuropathic pain) or persistent pain. In yet another embodiment, the control activity or expression can be selected amongst an established standard of KCC2 activity or expression; a corresponding level of KCC2 activity or expression determined in the subject at an earlier time; a corresponding level of KCC2 activity or expression determined in the subject when the subject is experiencing less pain (as above) or substantially no pain; or a corresponding level of KCC2 activity or expression

determined in a control subject experiencing less pain (as above) or substantially no pain. In an embodiment, the KCC2 activity may be determined as described above.

For example, intracellular chloride levels may be determined by administering, to a subject, an indicator compound (such as a compound indicative of chloride level) that is capable of contacting a CNS neural cell of that subject. Following the administration of the indicator compound, assessment of the *in vivo* signal associated with such indicator compound may be performed. In an embodiment, an indicator compound, such as a radionuclide (e.g. Thallium-201 (^{201}Tl), ^{99}Tcm-tetrofosmin, ^{99}Tcm-MIBI or ^{99m}Tc-HMPAO or chloride conjugates thereof) or a compound indicative of KCC2 expression (such as an immunodetection-based reagent (e.g. antibody, single chain antibody or Fab fragment directed against the KCC2 polypeptide)) may be employed. In yet another embodiment, the indicator compound, upon intravenous injection, may cross the blood-brain-barrier and accumulate in neurons of the CNS analogously to potassium, i.e. to reflect potassium levels. In another embodiment, the dose of such radionuclide (e.g. ^{201}Tl) may be about 100 MBq (3mCi). In yet another embodiment, the radionuclide (e.g. ^{201}Tl) may be injected 15-20 minutes prior to SPECT imaging. Following injection of the indicator compound, an imaging technique may be performed to assess the *in vivo* signal associated with the indicator compound. Such imaging techniques include, but are not limited to, single photon emission computed tomography (SPECT), positron emission tomography and/or magnetic resonance imaging. The imaging technique may enable the assessment of the *in vivo* signal of the indicator compound, such as the neural potassium gradient. Images can be obtained, for example, using gamma camera equipped with a high-resolution (5-7 mm) collimator and interfaced with a

dedicated computer system. In an embodiment, serial projection images can be aquired over a 180° arc. In yet another embodiment, the radionuclide (e.g. ^{201}Tl) retention by neurons can be expressed as a retention index (RI). The "retention index" as described herein is defined as:

$$\frac{\text{Delayed retention-early retention}}{\text{Early retention}} \times 100$$

In an embodiment, the "retention" of the retention index is herein defined as the amount of indicator compound (e.g. tracer or radionuclide) retained by a specific tissue at a certain time. In a further embodiment, the early retention is assessed before the delayed retention. In a further embodiment, the retention index is measured in a CNS tissue.

In an embodiment, the methods of diagnosis/prognostication noted above may be performed in conjunction with the therapeutic/prophylactic methods noted above, for preventing or treating pain associated with CNS dysfunction in a subject. Such a method thus comprises the diagnosis or prognostication of pain associated with CNS dysfunction and, in accordance with the diagnosis/prognosis, decreasing intracellular chloride levels in a CNS cell of the subject thereby to prevent or treat pain.

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Numeric ranges are inclusive of the numbers defining the range. In the claims,

the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The following examples are illustrative of various aspects of the invention, and do not limit the broad aspects of the invention as disclosed herein.

Throughout this application, various references are referred to describe more fully the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

EXAMPLES

Example 1: Methods

Nerve Injury. Briefly, peripheral nerve injury was induced by surgically implanting a polyethylene cuff (~2 mm long, 0.7 mm inner diameter) around the sciatic nerve of adult, male, Spague-Dawley rats as previously described (16). A group of rats also received sham surgery. Only animals that showed a gradual decrease in mechanical threshold (over 14-17 days) down to 2.0 g or less were used for further experiments.

Behavioural Testing. Thermal and mechanical threshold for nociceptive withdrawal reflexes were tested as previously described (17).

Slice preparation. Parasagittal slices (300-350 μm) of spinal cord were prepared from adult (>50 days old) male rats as previously described (9). Slices were continually superfused (2-3 $ml \cdot min^{-1}$) with artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 0.001 TTX (bubbled with 95% O_2

- 5% CO_2, pH~7.4); when measuring $GABA_A$/GlyR- mediated currents, 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 40 µM D2-amino-5-phosphonovaleric acid (APV) were added to block fast glutamatergic transmission.

Recordings. For perforated patch recordings, the pipette tip was filled with a solution containing (in mM): 130 cesium gluconate (CsGluc), 5 CsCl, 2 $MgCl_2$, 11 BAPTA Calcium chelator (buffer), 1 $CaCl_2$, 4 ATP, 0.4 GTP, 10 HEPES (pH~7.4). The pipette was back-filled with this same solution supplemented with 25 µg/ml gramicidin D (gramicidin stock was at 10 mg/ml in DMSO). Recordings in this mode were selected when access resistance was stable between 25-45 MΩ. For whole-cell voltage-clamp recordings, pipettes were filled with the above solution without gramicidin D. Similarly, whole-cell current-clamp recordings were performed using pipettes filled with the same intracellular solution as with voltage-clamp, except potassium methyl sulfate ($KMeSO_4$) was used to replace CsGluc. To clamp E_{anion} at 0 mV, CsGluc was replaced with 110 mM CsCl in the intracellular solution. All whole-cell recordings at E_{anion} = 0 mV were made at Vm = -60 mV in the presence of GluR-blockers. GABA was applied locally for 30-250 ms by pressure ejection through a patch micropipette. Data acquisition and analysis of PSCs was performed as previously described (9). All measurements are given as means ± SEM, except where indicated. Statistical significance was tested using Student's t-tests for comparison of mean values, chi-square tests for contingency tables, and mixed design ANOVAs (post-hoc – Tukey's HSD) for repeated measures.

Calcium Imaging. Slices were prepared from PNI and naïve rats as detailed above for electrophysiological analysis.

After 15 min incubation in ACSF, slices were loaded with 10 μM Fura-2-AM (a fluorometric calcium indicator, AM = acetoxymethyl) in HEPES-buffered saline (+10% DMSO) for 1 hour. Slices were washed for ~15 min with ACSF before being mounted in the recording chamber, where they continued to be superfused by ACSF (2-3 $ml \cdot min^{-1}$). $[Ca^{2+}]_i$ was fluorometrically measured using a Zeiss Axioscope equipped with epifluorescence optics. Images were acquired using a TILL Photonics monochromator coupled to a CCD camera and regions of interest (for ratioing) were drawn on clearly distinct neuronal cell bodies.

Immunoblotting. Horizontal slices (150 μm) of the SDH were made from the lumbar enlargement of both PNI and naïve adult rats. Tissue extracts were prepared by homogenizing the slices with a Teflon pestle in a buffer containing 0.32 M sucrose, 0.5 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetracetic acid (EDTA), 2.5 mM β-mercaptoethanol, and a cocktail of protease inhibitors (Complete™, Roche Diagnostics). Supernatants from 3,000 *g* (20 min) and 10,000 *g* (30 min) centrifugations were collected. Equal amounts of proteins (20 μg/lane) diluted in sample buffer were preheated at 37°C for 30 min, resolved by SDS-PAGE, and electroblotted onto nitrocellulose membranes. Membranes were blocked 30 min in 5% nonfat dry milk in TBST buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween-20) and incubated overnight at 4°C with a rabbit anti-KCC2 antibody (1:1000, Upstate Biotechnology). After several washes in TBST, membranes were incubated for 30 min at room temperature with peroxidase-labeled goat anti-rabbit antibody (1:2000). Chemiluminescent bands were detected using Super Signal Femto™ (Pierce Biotechnology). Digital images were

captured with the VersaDoc™ imaging system (BioRad) and data were analysed with Quantity One™ software (BioRad).

Oligodeoxynucleotides. KCC2 antisense and scrambled oligodeoxynucleotides, phosphorothioated at all positions were designed as previously described (18): antisense, 5'-TCTCCTTGGGATTGCCGTCA-3' (SEQ ID NO: 7; +59 relative to the ATG starting signal); scrambled, 5'-TCTTCTTGAGACTGCAGTCA-3' (SEQ ID NO: 8).

Intrathecal Injections. At least three days prior to drug administration, rats were anaesthetized with sodium pentobarbital (65 mg kg^{-1}) and a lumbar spinal catheter was inserted into the intrathecal space, as previously described (11). Briefly, a small opening was created at the cisterna magna, and a catheter was inserted into the subarachnoid space and caudally directed ~8 cm to the lumbar enlargement of the spinal cord. Upon recovery from surgery, lower body paralysis was induced via i.t. (intrathecal) lidocaine (2%, 30 µl) injection to confirm proper catheter localization. Only animals exhibiting appropriate, transient paralysis to lidocaine, as well a lack of motor deficits were used for behavioural testing. Following drug/vehicle administration, animals were sacrificed and their vertebral column dissected to visually confirm correct placement of the catheter. Drugs included DIOA (10-30 µg, in 0.9% NaCl, 10 % DMSO) and oligodeoxynucleotides (single doses of 2 µg at 0h, 12h & 24h; 0.9% NaCl). Behavioural testing was performed as above; normal (~15 g) mechanical threshold for withdrawal responses was confirmed in naïve rats prior to receiving drug or vehicle. At the doses used, none of the compounds produced motor disturbances or sedation as assessed by grasping,

righting and placing reflexes and behavioral observations (17).

Computer simulations. (see Figure 5) All simulations were performed with NEURON 4.3.1 using a compartment model of a generic spinal lamina I fusiform neuron with morphology and passive membrane properties based on (19). Dendrites bifurcated up to fourth order and an axon similar to that described in (19) were attached to the soma. Fast Na^+ and delayed rectifier K^+ currents based on (30) were inserted at 0.1 and 0.01 S/cm^2, respectively, in the soma and axon initial segment and nodes; voltage threshold for spiking was -49 mV. Two sets of inhibitory synapses were distributed randomly in the perisomatic region and four sets of excitatory synapses were more distal; each set was driven by an independent Poisson process at rates extrapolated from (31) and (32).

Electron microscopy. (see Figure 6) Tissue was prepared for ultrastructural analysis as previously described (35). Briefly, rats were perfused through the aortic arch with 0.9% NaCl followed by a fixative solution containing 4% paraformaldehyde (Sigma-Aldrich, Germany). After perfusion, spinal cords were removed, coronal blocks were dissected, then 60 µm thin sections were cut cryoprotected and freeze-thawed over liquid nitrogen and rinsed several times in phosphate buffer before incubation in the primary antiserum. After incubation in blocking solution containing 2% bovine serum albumin (BSA), sections were incubated in rabbit anti-KCC2 (1:500, Upstate Biotechnology, USA) for 48 hours at 4°C. After extensive washing, sections were incubated with 1 nm gold-conjugated anti-rabbit secondary antibody (1:250, Aurion) for 12 hours at 4°C followed by silver intensification (SE-EM, Aurion). Sections were treated with

0.5% OsO_4 (20 min), dehydrated in graded ethanol, then in propylene oxide and embedded in Durcupan ACM (Fluka). After ultra-sectioning (Ultracut™ UCT, Leica, Germany), specimens were examined using an electron microscope (Philips Tecnai 12, equipped with MegaView™ CCD camera). Non-consecutive (spacing> 3 µm) ultrathin sections were analyzed in the electron microscope. Boutons with synaptic profiles were randomly selected and analyzed in laminae I & II and white matter for the expression of the KCC2 protein (36).

Intrathecal administration of K-252a. (see Figure 8) K-252a was prepared in 25 ul of 0.9% NaCl solution containing 10% DMSO. Intrathecal catheterization was performed by creating a small opening at the cisterna magna, and inserting P10 polyethylene tubing into the subarachnoid space -- caudally directed ~8 cm to the lumbar enlargement of the spinal cord.

Example 2: Results

Peripheral neuropathy was induced in rats by chronically constricting the sciatic nerve (Fig. 1a). To test whether the hyperexcitability (sensitization) of SDH neurons that follows peripheral nerve injury (PNI) is associated with modification of the anion gradient (∇_{anion}), anion reversal potential (E_{anion}) was measured using gramicidin-perforated patch clamp recording. This technique avoids disrupting the intracellular anion concentration (8). Responses to exogenous GABA application showed that the anion reversal potential (E_{anion}) of lamina I (LI) neurons taken from PNI rats was -49.0 ± 2.3 mV (range: -40 to -62.2 mV, n = 9) compared to -72.6 ± 3.5 mV (range: -63.0 to -79.9 mV, n = 5; $p < 0.005$) in LI neurons from naïve rats (Fig. 1b-d). Resting membrane potential was not significantly different between

PNI (−62 ± 4 mV, n = 7) and naïve rat LI neurons (−61 ± 2 mV, n = 16; p> 0.1). Spontaneous and evoked postsynaptic currents (PSCs), recorded from PNI rat LI neurons in the presence of fast glutamate receptor (GluR) blockers were also inward (depolarizing from rest), their mean reversal potential increasing by 16.1 mV relative to that in lamina I neurons from naïve rats (n = 6, PNI; n =4, naïve). It was then investigated whether other properties of synaptic transmission were altered in the SDH after PNI. Inhibitory miniature PSCs (mPSCs) in LI neurons from naïve rats are mediated by glycine receptors (GlyRs) alone despite GABA and glycine corelease from local inhibitory interneurons (9; Fig.2a). While GluR-mediated mPSCs were unaffected by PNI (Fig.2b), in all cells tested from PNI rats, a population of outward mPSCs at 0 mV persisted in the presence of the GlyR antagonist strychnine (up to 1 μm; n = 4). These remaining mPSCs were mediated by $GABA_AR_S$, as they were blocked by bicuculline (10 μM) and displayed prolonged decay kinetics compared to the GlyR-mediated component ($\tau_{D(GABA_AR)} = 34.0 \pm 2.9$ ms, n = 5, vs. $\tau_{D(GlyR)} = 11.3 \pm 1.3$ ms, n = 6;p < 0.01; Fig. 2C).

Kinetic analysis further revealed that the decay phase of 36.9 ± 2.3% of mPSCs followed a dual exponential function ($\tau_{D1} = 7.5 \pm 2.0$ ms and $\tau_{D2} = 51.3 \pm 7.9$ ms; n = 6; Fig.2c). These events possessed both a $GABA_AR$ and a GlyR-mediated component, as either strychnine or bicuculline could lead to the abolition of their respective components (n = 4). Therefore, in parallel with the collapsed ∇_{anion}, PNI caused reorganization at LI synapses thereby unmasking $GABA_AR$-only and mixed $GABA_AR$/GlyR-mediated mPSCs, in addition to those mediated by GlyRs alone. This synaptic organization is similar to that observed in immature LI-II neurons (9). The net effect of this synaptic switch is that it yielded a

population of quantal synaptic events with significantly longer decay kinetics.

To examine the function of the PNI-induced $GABA_AR$-mediated contribution to mPSCs, we analysed both the peak conductance and the frequency of mPSCs. This was performed using CsCl-filled pipettes to clamp the E_{anion} at 0 mV in both LI neurons taken from PNI and naïve rats to prevent biased detection of mPSCs resulting from changes in driving force. Peak conductance of GlyR-only mPSCs recorded in LI neurons taken from PNI rats was significantly smaller (~2-fold) than that recorded from naïve rat LI neurons (Fig. 2d). The addition of $GABA_AR$-mediated events in the PNI condition, however, partially compensated the decrease in GlyR-only conductance. The peak conductance of GluR-mediated quantal events was not significantly different between LI neurons taken from naïve and PNI rats (Fig.2d).

Factoring together the changes in peak conductance, kinetics, and driving force, the net charge carried by GlyR-only mPSCs at resting membrane potential in LI neurons taken from PNI rats was nearly 3-fold smaller than that in naive rats (Fig. 2e). With the contribution of $GABA_ARs$, however, the net charge carried by mPSCs in PNI rats rose back to that mediated by GlyRs in naïve rats. This result suggests that, although equivalent in magnitude, hyperpolarizing charge in naïve LI neurons was carried by GlyR-mediated mPSCs alone, whereas depolarizing charge was transferred predominantly via $GABA_ARs$ in PNI rat LI neurons, due to the prolonged decay kinetics of $GABA_AR$-mediated mPSCs.

The frequency of GlyR-only mPSCs recorded in LI neurons from PNI rats was observed to be significantly less (0.13 ± 0.04 Hz, n = 5) than that for GlyR-only mPSCs in naive rat LI neurons (0.18 ± 0.04 Hz, n = 6; $p < 0.05$; Fig. 2f). As with peak conductance, however, the addition of the

$GABA_AR$-mediated mPSCs compensated the PNI-induced decrease in frequency (0.22 ± 0.10 Hz, n = 4, for all $GABA_AR$ and/or GlyR-mediated events combined; p > 0.5). In contrast, there was no significant change in the frequency of GluR-mediated events in LI neurons isolated from PNI rats (1.51 ± 0.90 Hz, n = 9) compared to LI neurons from naïve rats (0.82 ± 0.40 Hz, n = S; p> 0.3; Fig.2f).

If depolarizing $GABA_AR$/GlyR-mediated postsynaptic currents exert a net excitatory influence in PNI LI neurons, they should directly evoke action potentials, and consequently lead to Ca^{2+} influx . To test this hypothesis, we employed Ca^{2+}-imaging using fura-2-am loaded LI neurons in slice to obtain a large data set. Administration of exogenous GABA to neuronal somata caused a significant increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in 19% of LI neurons (n = 53; Fig. 3a,c) lying ipsilateral to the site of PNI. This represents a seven-fold increase compared to LI neurons found in naïve and/or contralateral dorsal horn, where an increase in $[Ca^{2+}]_i$ to GABA application was observed in only 1 of 37 neurons tested (Fig. 3b,c). These responses were blocked by bicuculline (10 µM; n = 5) and by the voltage sensitive sodium channel blocker tetrodotoxin (TTX; 1 µM; n = 31). We then further confirmed electrophysiologically that applied GABA and synaptically elicited anionic postsynaptic potentials can directly evoke action potentials (Fig. 3d,e). These results indicate that postsynaptic anion fluxes can cause net excitation in lamina I neurons in PNI rats.

We then compared KCC2 protein levels by immunoblotting on horizontal slices of SDH. The KCC2 expression level in the lumbar SDH ipsilateral to the PNI was significantly reduced (>2-fold) relative to the side contralateral to the injury (Fig. 3f). In naïve rats, there was no significant difference between the two sides (n = 3).

If a decrease in the expression of the KCC2 exporter leads to an increase in neuronal $[Cl^-]_i$ and, in turn, $GABA_AR$-mediated depolarization, a pharmacological blockade of the KCC2 exporter in LI neurons from naïve rats should have the same effect. To test for this possibility, we bath applied the selective KCC2 blocker DIOA (30 µM) to naïve spinal slices. As in the PNI condition, GABA application in the presence of DIOA caused an increase in $[Ca^{2+}]_i$ in 30% of naïve LI neurons tested (Fig. 3b,c). To assess whether the empirically determined changes in $GABA_AR$/GlyR-mediated postsynaptic control were sufficient to account for the central sensitization which follows PNI, we simulated *in vivo* conditions using a biophysically realistic neuron model (Fig. 5). The simulation confirmed that, after PNI, the extent of LI neuronal sensitization varied as a function of their E_{anion}, ranging from slight disinhibition to a net hyperexcitation.

To test whether this hyperexcitability (sensitization) would result in a decrease in the stimulus threshold to evoke a nociceptive withdrawal reflex, we administered DIOA (15-30 µg) directly to the lumbar enlargement of the spinal cord in intact rats via an intrathecal catheter. DIOA caused a rapid and reversible decrease in nociceptive threshold to both mechanical and thermal stimuli (Fig. 4a-b). A similar decrease in nociceptive threshold was further obtained via selective knock-down of the exporter using spinal administration of an antisense oligodeoxynucleotide against KCC2 mRNA (Fig. 4e), further confirming the functional impact of KCC2 dowregulation.

As shown in Figure 7, we demonstrate that in lamina I neurons taken from rats with peripheral neuropathy, the transmembrane anion reversal potential (E_{anion}) is

significantly more depolarized than that in lamina I neurons taken from naïve rats. The anion (bicarbonate and chloride) reversal potential (E_{anion}) of recorded from lamina I neurons taken from naïve rats was significantly less than that recorded from the lamina I neurons of rats that had received a peripheral nerve injury (PNI). Bath application of both BDNF (50 ng/ml; N/BDNF) and NGF (50 ng/ml; N/NGF) caused the E_{anion} recorded from naïve rat lamina I neurons to become significantly depolarized, indicating a collapse of the transmembrane anion gradient. Alternatively, bath application of the TrkB antagonist K-252a (200 nM; P/K252a) to lamina I neurons taken from a PNI rat caused a hyperpolarization of the E_{anion} to a level similar to that observed in lamina I neurons taken from naïve rats. All E_{anion} values were confirmed using gramicidin-D perforated-patch voltage-clamp recordings. This depolarized E_{anion} is the result of a decreased expression of the KCC2 cotransporter in the lamina I neurons taken from neuropathic rats, as noted above. In lamina I neurons from naïve rats, it is further shown herein that the E_{anion} may be depolarized significantly via the perfusion of the growth factors NGF and BDNF, suggesting that these growth factors may decrease the function and/or expression of the KCC2 protein in the superficial dorsal horn. Alternatively, blocking the BDNF receptor, TrkB, in lamina I neurons taken from neuropathic rats using the protein kinase inihibitor K-252a, is shown herein to reverse the depolarization of the E_{anion}, returning this value to a level similar to that observed in lamina I neurons taken from naïve rats. Further, as shown in Figure 8, intrathecal administration of the receptor tyrosine kinase inhibitor K-252a (6 nM) (but not vehicle injection alone) resulted in an increase in the threshold for tactile nociceptive withdrawal in rats that had received peripheral

nerve injury. K-252a can thus reverse the hyperalgesia/allodynia after its development following peripheral nerve injury. K-252a did not produce any motor disturbances or sedation as assessed by grasping, righting and placing reflexes and behavioral observations. It is envisioned that this inhibitor reactivates the KCC2 cotransporter in lamina I neurons taken from neuropathic rats by blocking phosphorylation, perhaps at a protein tyrosine kinase site on the transporter or on its transcription factors (or other regulatory substrate).

The results herein show that the painful neuropathy that follows PNI can be explained by a downregulation of the KCC2 exporter and the resultant shift in the ∇_{anion} in spinal LI neurons. They also demonstrate that such a modification of ∇_{anion} in adult animals can occur in a neuron transsynaptic to an injury site. Previous efforts to identify a substrate underlying the hyperexcitability characteristic of peripheral neuropathy have focussed on measuring changes in number of GABAergic interneurons, GABA content or $GABA_AR$ expression. The results have been contradictory (3-6). The findings presented herein provide a new avenue to understand such mechanisms of disinhibition. The conversion of the $GABA_AR$/GlyR-mediated postsynaptic action via a shift in ∇_{anion} provides a mechanistic basis for central sensitization, including increases in neuronal responsiveness and number of excitatory inputs.

A critical feature of the spinal cord is that it employs two very distinct GABAergic inhibitory mechanisms: GABAergic control of the central terminals of sensory fibres already involves a depolarizing mechanism (39), in contrast to dorsal horn cells where GABAergic inhibition involves hyperpolarization. Thus, the change in KCC2 expression reported here affects the polarity of GABA action in only one

of the two inhibitory mechanisms controlling sensory input. This is confirmed by the fact that primary afferents lack expression of KCC2 (Fig.4f, g; see also Fig. 6). GABA/glycine-mediated depolarization may also serve as a gating mechanism to enable excitation via voltage sensitive Ca^{2+} channels (VSCCs) and NMDA receptor/channels (10). Ca^{2+} influx via these channels is thought to be critical for the sensitization of spinal neurons (11). Indeed, blocking these Ca^{2+} channels in humans by drugs such as gabapentin and ketamine has proven highly efficacious in the treatment of neuropathic pain (12-14). However, use of Ca^{2+} channel blockers, particularly ketamine and other NMDA antagonists, is associated with many undesirable side effects (14, 15).

Example 3 – *In vitro* TrkB-dependent modulation of KCC2

Parasaggital slices (250-300 microm) were made from the dorsal horn of naïve rats or PNI rats. Slices were continually perfused with an oxygenated Ringer's solution and were permitted to equilibrate for at least 1.5 hours prior to manipulation. Unless otherwise specified, slices were further perfused with 10 microM CNQX, a blocker of non-NMDA ionotropic glutamate receptors. Recordings were made from visually-identified lamina I neurons using gramicidin-D perforated-patch or whole-cell voltage-clamp recordings. In both cases, pipettes were filled with an intracellular solution containing either potassium methyl sulphate or cesium gluconate as the major ionic species. E_{anion} was measured by applying a series of brief (5-10 ms) applications of exogenous GABA to the soma of the neurons of interest; by manipulating the membrane potential of the neuron, the point at which GABA elicited neither an inward nor an outward anion current was taken as E_{anion}. All measurements of membrane

potential were corrected for liquid junction potential, pipette offset, and resistances.

As shown in Figure 12, both brain-derived neurotrophic factor- (BDNF; 50 ng/ml in bath) mediated activation of TrkB and nerve growth factor- (NGF; 50 ng/ml in bath) mediated activation of TrkA caused a significant depolarization of the anion reversal potential (E_{anion}) in lamina I neurons taken from naïve rats.

Using slices taken from peripheral nerve injured (PNI) rats, where the E_{anion} is chronically depolarized, application of various inhibitors of components of an intracellular pathway coupled to TrkB receptors were shown to cause a significant hyperpolarization of the E_{anion} (bicarbonate and chloride), to levels similar to that observed in slices taken from naïve rats (Figure 13). Agents that rendered this effect included, but are not limited to, an antibody directed against TrkB, (anti-TrkB-IgG 1 μg/ml in bath); K-252a, an inhibitor of TrkA/B autophosphorylation (200 nM in bath); H-89, a membrane-permeable inhibitor of cyclic AMP-dependent kinase (PKA); 15 μM in bath); and KN-93, a membrane permeable inhibitor of calmodulin-dependent kinase II and IV (5 μM in bath).

Example 4 - TrkB-dependent modulation of nociceptive threshold *in vivo*.

All drugs used for local spinal delivery via intrathecal catheter were dissolved in 0.9% NaCl with or without 10% v/v DMSO. Intrathecal catheterization was performed by creating a small opening at the cisterna magna, and inserting a short P10 polyethylene tube into the subarachnoid space, caudally directed ~ 8 cm to the lumbar enlargement (L4-5) of the spinal cord. No drug administered

produced motor disturbances or sedation, as assessed via analysis of grasping, righting and placing reflexes and other behavioral observations. Von Frey testing was used to assess the 50% withdrawal threshold to mechanical stimulation as previously described (41). All experiments were performed on intact, adult Sprague-Dawley rats.

Local spinal delivery of various agents using an intrathecal catheter led to the identification of several compounds that either effect a reduction of nociceptive threshold for tactile stimulation in naïve rats, or raise the nociceptive threshold in PNI rats.

Local spinal delivery of either an adenovirus transducing BDNF (Figure 14) or human recombinant BDNF (10 µg/day x 6 days), but not of an adenovirus transducing the green-fluorescent protein, caused a significant decrease in the nociceptive threshold for mechanical stimulation in naïve rats. Likewise, intrathecal delivery of human recombinant NGF (Figure 15; 10 µg/day x 6 days) to naïve rats caused a very similar decrease in the said nociceptive threshold.

On the other hand, serial administration of antibody directed against TrkB (anti-TrkB-IgG 12 µg/2 hrs x 3) via intrathecal catheter to PNI rats effected a significant increase in nociceptive threshold to mechanical stimulation (Figure 16). Local spinal delivery of the PKA inhibitor H-89 (380 nmol) also caused an increase in the nociceptive threshold (Figure 17).

Throughout this application, various references are cited, which describe more fully the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

REFERENCES

1. Melzack,R. & Wall,P.D. (1965) *Science* 150:971-979.
2. Woolf,C.J. & Salter,M.W. (2000) *Science* 288:1765-1769.
3. Kontinen,V.K., et al. (2001) *Anesthesiology* 94:333-339.
4. Moore,K.A. et al. (2002) *J. Neurosci* 22:6724-6731.
5. Somers,D.L. & Clemente,F.R. (2002) Neurosci Lett 323:171-174.
6. Polgar,E., et al. Soc Neurosci Abstr 28, 655.3. 6-11-2002.
7. Light,A.R. The initial processing of pain. Karger, Basel (1992).
8. Ebihara,S., et al. (1995) *J Physiol* 484 (Pt 1), 77-86.
9. Keller,A.F., et al. (2001) *J Neurosci* 21:7871-7880.
10. Staley,K.J., et al. (1995) *Science* 269:977-981.
11. Coderre,T.J. & Melzack,R. (1992) *J Neurosci* 12:3665-3670.
12. Serpell,M.G. (2002) *Pain* 99:557-566.
13. Rabben,T., et al. (1999) *J. Pharmacol. Exp. Ther.* 289:1060-1066.
14. Martin,T.J. & Eisenach,J.C. (2001) *J. Pharmacol. Exp. Ther.* 299:811-817.
15. Farber,N.B., et al. (2002) *Mol. Psychiatry* 7:726-733.
16. Mosconi,T. & Kruger,L. (1996) *Pain* 64:37-57.
17. Coderre,T.J. & Van,E., I. (1994) Pain 59:345-352.
18. Rivera,C. et al. (1999) *Nature* 397:251-255.
19. Prescott,S.A. & de Koninck,Y. (2002) *J Physiol* 539:817-836.
20. Payne,J.A., et al. (1996) *J. Biol. Chem.* 271:16245-16252.
21. Mount,D.B. *et al.* (1999) *J. Biol. Chem.* 274:16355-16362.
22. Kelsch,W. *et al.* (2001) *J Neurosci* 21:8339-8347.
23. Strange,K., et al. (2000) *Am. J. Physiol Cell Physiol* 279:C860-C867.

24. Shen,M.R. *et al.* (2001) *Proc. Natl. Acad. Sci. U. S. A* 98:14714-14719.

25. Gulyas,A.I., et al. (2001) *Eur. J. Neurosci.* 13:2205-2217.

26. Payne,J.A. (1997) *Am. J. Physiol* 273:C1516-C1525.

27. Flatman, P.W., et al. (1996) *Am. J. Physiol* 271:C255-C263.

28. Howard,H.C. *et al.* (2002) *Nat. Genet.* 32:384-392.

29. Sung,K.W., et al. (2000) *J. Neurosci.* 20:7531-7538.

30. Mainen,Z.F., et al. (1995)*Neuron* 15:1427-1439.

31. Traub,R.J. & Miles,R. Neuronal Networks of the Hippocampus. Cambridge, Cambridge, U.K. (1991).

32. Narikawa,K., et al. (2000) *J Neurophysiol* 84:2171-2174.

33. Furue,H., et al. (1999) *J Physiol* 521:529-535.

34. Ribeiro-da-Silva, A. & Coimbra,A. (1982) *J. Comp Neurol*. 209, 176-186.

35. Ribeiro-da-Silva,A. The Rat Nervous System. Paxinos,G. (ed.), pp. 47-59 (Academic Press, Sydney, Australia, 1995).

36. Sik,A., et al. (2000) *Neuroscience* 101, 51-65.

37. Gulyas,A.I., et al. (2001) *Eur. J. Neurosci*. 13, 2205-22 17.

38. Delpire, E. & Mount, D.B. (2002) *Annu. Rev. Physiol*. 64:803-403.

39. Woo, N-S., et al. (2002) *Hippocampus* 12:258-268.

40. Rudomin,P. & Schmidt,R.F. (1999) *Exp Brain Res* 129:1-37.

41. Chaplan et al., (1994) *J. Neuroscience Methods* 53:55-63.

42. Jarolimek at al. (1999) *J. Neuroscience* 19: 4695-704.